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A functional benchmark dose analysis

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Transcriptomic effects of tributyltin (TBT) in zebrafish eleutheroembryos. A functional benchmark dose analysis.

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Graphical abstract

Highlights

- 3238 genes were differentially expressed after the exposure to tributyltin (TBT).
- Transcriptomic point of departure occurred at concentrations 1/7 of the macroscopic ones.
- Steroid, cholesterol and vitamin D₃ related pathways were the most affected.
- Lipid, general stress and DNA damage related pathways were altered at higher doses.
- Usefulness of functional benchmark dose approach for risk assessment is highlighted.

Abstract

Exposure to the antifouling tributyltin (TBT) has been related to imposex in mollusks and to obesogenicity, adipogenesis and masculinization in fish. To understand the underlying molecular mechanisms, we evaluated dose-response effects of TBT (1.7-56 nM) in zebrafish eleutheroembryos transcriptome exposed from 2 to 5 days post-fertilization. RNA-sequencing analysis identified 3238 differentially expressed transcripts in eleutheroembryos exposed to TBT. Benchmark dose analyses (BMD) showed that the point of departure (PoD) for transcriptomic effects (9.28 nM) was similar to the metabolomic PoD (11.5 nM) and about one order of magnitude lower than the morphometric PoD (67.9 nM) or the median lethal concentration (LC₅₀: 93.6 nM). Functional analysis of BMD transcriptomic data identified steroid

metabolism and cholesterol and vitamin D₃ biosynthesis as the most sensitive pathways to TBT (<50% PoD). Conversely, transcripts related to general stress and DNA damage became affected only at doses above the PoD. Therefore, our results indicate that transcriptomes can act as early molecular indicators of pollutant exposure, and illustrates their usefulness for the mechanistic identification of the initial toxic events. As the estimated molecular PoDs are close to environmental levels, we concluded that TBT may represent a substantial risk in some natural environments.

Abbreviations

ANOVA-PLS: Analysis of Variance - Partial Least Square

BMD: benchmark dose

BMDL: lower limit of the benchmark dose

BMDU: upper limit of the benchmark dose

DBT: dibutyltin

DEGs: differentially expressed genes

dpf: days post-fertilization

EC₅₀: half maximal effective concentration

EDC: endocrine disrupting chemical

hpf: hours post fertilization

KEGG: Kyoto Encyclopedia of Genes and Genomes

LC₅₀: median lethal concentration

LOAEC: lowest observed adverse effect concentration

LOECs: lowest observed effect concentrations

MIE: molecular initiating event

MoA: mode of action

NOECs: no observed effect concentrations

PAM: partition around medoids

PoD: point of departure

PPAR: peroxisome proliferator-activated receptors

RIN: RNA integrity number

RXR: retinoid X receptors

TBT: tributyltin

VDR: vitamin D receptors

Keywords: tributyltin, zebrafish, endocrine disruption, RNA-Seq, benchmark dose

1. Introduction

Organotin compounds are organometallic chemicals based on several hydrocarbon chains which form direct Sn-C bonds with a central tin atom. Among them, TBT (tributyltin) compounds ($\text{Sn}(\text{C}_4\text{H}_9)_3^+ \text{X}^-$) show up as an important class of organotins,

consisting in three butyl groups covalently bonded to a Sn^{4+} center atom (Davies, 2004). They possess several applications as disinfectants, microbiocides, fungicides and wood preservatives, and are used in water cooling towers, textiles products, sponges, foams, paper and building materials (U.S. Environmental Protection Agency, 2008). Precisely, its biocides properties triggered its worldwide use in anti-fouling paints in boats as a copolymer (Antizar-Ladislao, 2008; Yebra et al., 2004). Despite of the relative low solubility of organotins in water, they are able to reach the environment through a hydrolysis reaction of its copolymer that release tributyltin (TBT; $(\text{C}_4\text{H}_9)_3\text{Sn}^+$) (IMO, 2002; Takahashi, 2009). Despite the ban on organotin compounds and their inclusion in the Rotterdam Convention (Davies, 2004; IMO, 2002; Takahashi, 2009), their long half-life in sediments and their progressive release (Champ and Seligman, 1996), their current production in some countries, the poor regulation in water dumpings (Wang et al., 2019) and illegal uses (Bray, 2006; Ramaswamy et al., 2004) lead to the presence of concentrations high enough to be a matter of concern in different parts of the world (Castro and Fillmann, 2012; Langston et al., 2015). Total organotin concentrations range values of 1.76 - 486.62 ng Sn/g (dry weight) in Asian harbors (Wang et al., 2019), 43.9 - 63.5 ng Sn/L in some Asian rivers or 0.01-1.9 $\mu\text{g Sn/L}$ (equivalent to 0.37-0.53 nM and to 0.08-16.0 nM of TBT, respectively) in coastal waters around the world (Li et al., 2019).

TBT is very toxic for marine organisms and shows endocrine disrupting properties, like promoting dysregulation of the synthesis of steroid hormones (Nakanishi, 2008). It can exert shell thickening and aberrant morphogenesis (imposex) in molluscs (Newman and McIntosh, 1993; Santillo and Langston, 2001) and obesogenicity, adipogenesis and masculinization in fish (Heindel and Blumberg, 2019; Lyssimachou et al., 2015; Santos et al., 2006). Although TBT is not usually included in the endocrine disrupting chemicals (EDCs) lists (ECHA (European Chemicals Agency), 2019; European Commission, 2007; Hass et al., 2018), many international agencies have recognized its EDC properties (Bergman et al., 2013; Demeneix and Slama, 2019; Gore et al., 2014). It has been proposed that the mode of action (MoA) of TBT is via covalent binding with retinoid X receptors (RXR) (le Maire et al., 2009). Although its effects at phenotypical, organismal and transcriptomic levels have been explained in other species (Baker and

Hardiman, 2014; Cruz et al., 2015b; Zhang et al., 2018), not much is known on its transcriptomic effects in zebrafish eleutheroembryos. This fish species is considered an excellent vertebrate model for human and environmental toxicology, including endocrine disruption, thanks to its easy rearing and to the characteristics of their free-swimming late embryonic stages (Kimmel et al., 1995a; Parichy et al., 2009; Raldúa and Piña, 2014; Scholz and Mayer, 2008; Segner, 2009; Stegeman et al., 2010; Strähle et al., 2012).

Recent developments on high-throughput next generation sequencing (HT-NGS) technologies, and especially RNA-Seq, has allowed to perform *in toto*, untargeted and integrative analyses of the effects of several EDCs in many organisms, including zebrafish (Caballero-Gallardo et al., 2016; Martínez et al., 2019b; Qian et al., 2014; Reuter et al., 2015; Wang et al., 2009; Zhao et al., 2014). Moreover, it has been observed that the phenotypical observations as well as the metabolomic alterations produced by pollutants can be linked to transcriptomic dysregulation (Lee et al., 2017; Orlando et al., 2004; Ortiz-Villanueva et al., 2018), which usually is the first biological response to EDC exposure (Martínez et al., 2019b; Tu et al., 2019). The benchmark dose (BMD) concept defines the concentration that induces physiological changes that can be associated to an adverse effect. This concept has been applied to risk assessment since early 1980s (Davis et al., 2011; Filipsson et al., 2003), but only recently has been extended to transcriptomic responses, in order to: 1) detect the most sensitive pathways to EDC exposures using transcriptomic datasets (Wang et al., 2018), which could be used to identify primary response biomarkers of EDC exposures; 2) integrate data from different biological levels (Martínez et al., 2019b); and 3) determine the point of departure (PoD) of different structurally similar chemicals (Tu et al., 2019). However, the majority of studies evaluating transcriptomic points of departure have been done in adult animals and more studies based on early-stage exposures are needed (Pagé-Larivière et al., 2019).

In the present work, we exposed zebrafish eleutheroembryos from 2 to 5 days post-fertilization (dpf) to several TBT concentrations in a dose-response manner. We then studied the effects at the transcriptomic level using the benchmark dose approach,

and analyzed the individual gene responses according to their functional annotation. Finally, we used this information, as well as published data from previous studies on the effects of TBT at morphological and metabolical levels (Martínez et al., 2019a; Ortiz-Villanueva et al., 2018) to identify the different regulatory mechanisms responsible for the multiple toxic effects of TBT in zebrafish.

2. Materials and methods

2.1. *Animals and rearing conditions*

Zebrafish eggs were obtained by natural mating of adult individuals (wild-type *Danio rerio*, 12-18 months old) which were fed twice per day with dry flakes (TetraMin, Tetra, Germany). Mating was carried out in 4-L breeding tanks with 6 males and 3 females (2:1 proportion). A mesh bottom was placed for egg collection. Adults, eggs and embryos were maintained under standard conditions (28.0 ± 1.0 °C, photoperiod of 12L:12D) and kept in fish water which was composed of Instant Ocean (90 µg/ml, Aquarium Systems, Sarrebourg, France) and $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (100 µg/ml, Sigma-Aldrich, St.Louis. MO- USA), dissolved in reverse-osmosis purified water. Eggs were collected at 2 hours post fertilization (hpf), rinsed and checked for fertilization. Embryos were placed in 6 multi-well plates at a density of 3.3 embryos/ml (10 individuals per well, in 3.0 ml) in clean fish water until the start of the exposures. Treatment assignment in the plates was randomized to avoid any possible bias. From now on, we will refer to hatched embryos that have not reach the stage of self-feeding (about 120 hpf in our conditions) as eleutheroembryos. All experimental procedures were carried out under the license DAMM 7669, 7964 from the competent authority in accordance with the institutional guidelines. They were approved by the Institutional Animal Care and Use Committees of the Research and Development Centre (CID) of the Spanish National Research Council (CSIC).

2.2. *Zebrafish eleutheroembryos exposures to TBT*

Tributyltin chloride (TBT, CAS-RN: 1461-22-9) was provided by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C. During the exposure period, fresh experimental solutions were prepared every

day by dilution of the stock solutions in fish water (final DMSO concentration: 0.2% (v/v)). With the aim to avoid possible molecular events directly related with cellular/organism death processes, the exposure was set up using as a maximum TBT concentration the previously reported lowest observed adverse effect concentration (LOAEC) in terms of survival, hatching or swim bladder inflation rates (100 nM) (Martínez et al., 2019a). Based on that, we chose to expose zebrafish eleutheroembryos to nominal concentrations of 0 (control; 0.2% DMSO), 3, 30 and 100 nM of TBT from 2 to 5 dpf. Exposures were carried out from 2 to 5 dpf to ensure that many of the endocrine systems were developed or under development (Löhr and Hammerschmidt, 2011) and also to avoid toxic effects at early embryonic developmental processes to focus the analysis on already differentiated cells and tissues. This exposure window has been used in the past in our laboratory (Martínez et al., 2019a, 2019b, 2018; Ortiz-Villanueva et al., 2018) and it has been recognized as a good experimental window to study the effect of EDCs in zebrafish embryos by other researchers (Jarque et al., 2019). The present exposures occurred simultaneously with the ones from which metabolomic and morphometric data was obtained (Martínez et al., 2019a; Ortiz-Villanueva et al., 2018). TBT is stable in solution for at least some days (Cruz et al., 2015a; Jordão et al., 2016). Exposure medium was daily renewed and chemical analysis of TBT concentrations were measured as total Sn in the aqueous solutions by inductively coupled plasma mass spectrometry (ICP-MS) in laboratory ICP-MS/AES of IDAEA (Institute of Environmental Assessment and Water Research, Spain). From now on, we will refer to measured concentrations (nominal and measured TBT concentrations are reported at **supplementary table ST1**).

Anatomical development of embryos was followed daily during the exposure (Kimmel et al., 1995b) and survival (3, 4 and 5 dpf), hatching (3, 4 and 5 dpf) and swim bladder inflation rates (4 and 5 dpf) were recorded (210 embryos of each experimental condition were assessed by visual observation; **supplementary table ST1**). Replicates for each TBT condition (10 zebrafish eleutheroembryos per replicate) were collected, snap-frozen in dry ice and stored at -80 °C until further steps.

2.3. RNA extraction, library construction and high-throughput sequencing

Total RNA was extracted from independent pools of 10 eleutheroembryos using AllPrep DNA/RNA Mini Kit (Qiagen, CA, USA) as described by the manufacturer. RNA quality and concentrations were assessed with a NanoDrop® 8000 UV-Vis Spectrophotometer (Thermo Scientific). RNA preparations of three high quality replicates per condition were sent to the National Center for Genomic Analysis (CNAG, Barcelona, Spain) for high-throughput sequencing (RNA-Seq). Their quantity and quality were assessed by a Qubit® RNA BR Assay kit (Thermo Fisher Scientific) and RNA 6000 Nano Assay on a Bioanalyzer 2100 (Agilent Technologies), respectively. RNA concentrations ranged between 160 and 514 ng/μL, RNA integrity numbers (RIN) were higher than 8.5 and samples were free of genomic DNA. RNA-Seq libraries were prepared with the KAPA Stranded mRNA-Seq Kit Illumina® Platforms (Kapa Biosystems) with minor modifications. Briefly, 500 ng of total RNA as input material was poly(A) enriched with oligo-dT magnetic beads and fragmented (resulting RNA fragment size: 80 - 250 nt; major peak: 130 nt). The synthesis of the second strand cDNA was performed with dUTP instead of dTTP, to enhance strand specificity. A 3' adenylation and ligation of indexed Illumina adapters were performed over the blunt-ended double-stranded cDNA. An enrichment step (15 PCR cycles) was carried out in the ligation product and validated on an Agilent 2100 Bioanalyzer with the DNA 7500 kit. Each final library was sequenced using TruSeq SBS Kit v3-HS (paired-end mode; 2x76bp as read length). We generated on average 42 million paired-end reads for each sample in a fraction of a sequencing lane on HiSeq2000 (Illumina). Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA 1.13.48) and FASTQ sequence files were generated by the sequencing analysis software CASAVA. For each sample, more than a 98% of the obtained reads mapped properly to the reference genome (GRCz10). Most of them mapped to exonic regions and to protein-coding genes, with a total of 24,420 genes detected per sample. A full description of mapping quality statistics can be found in **supplementary material**.

2.4. Data analysis

2.4.1. Survival, hatching and swim bladder inflation rates

To determine differences in the survival, hatching and swim bladder inflation of the

eleutheroembryos triggered by the TBT exposure, individual non-parametric Mann-Whitney-Wilcoxon tests against each control group (one per day) were performed over the percentages obtained (5 replicates per condition, 10 eleutheroembryos per replicate, $p \leq 0.05$). EC_{50} values (half maximal effective concentrations) were calculated by interpolation on the fitted curves (Hill equations with variable slope). GraphPad Prism version 8.1.2 (GraphPad Software, San Diego, California USA, www.graphpad.com) was used to perform all the statistical analyses and associated graphs.

2.4.2. RNA-Seq transcriptomic data

Reads obtained by RNA-Seq were aligned to the Danio Rerio reference genome (GRCz10) using the STAR software version 2.5.1b (Dobin et al., 2013). RSEM software version 1.2.28 (Li and Dewey, 2011) was used for the quantification of the genes annotated in GRCz10.84 (using default parameters). Data transformation was performed with the DESeq2 (v.1.10.1) R package (Love et al., 2014), which assumed that most genes are not differentially expressed. Afterwards, data was centered and scaled to the control set, log2 transformed and smoothed by loess function by *stats* package in R (R_Development_Core_Team, 2008) considering each TBT concentration (including control group) as a class. Statistical analysis to determine the differential expressed genes (DEGs) between all experimental conditions (adjusted p value < 0.05) was carried out by a ANOVA-PLS (Analysis of Variance-Partial Least Square) test using the *lmdme* package in R v. 1.0.136, R Core Team (Fresno et al., 2014). Briefly, this analysis consisted in a decomposition of the transcriptomic data matrix of all samples (normalized transcript values) through a linear model that considers the experimental design (TBT treatments) and builds a regression between the matrices obtained in the first linear decomposition (X) and a vector (y) that defined the class membership of the samples (control, 1.7, 17 and 56 nM). This analysis allowed to identify 3238 differentially expressed genes (DEGs) that significantly described best the differences between groups ($p \leq 0.05$). Subsequently, hierarchical and PAM (partition around medoids) clustering analyses were both performed over the DEGs and plotted using the packages *gplots*, *fpc*, and *cluster* in R (Hennig, 2014; Maechler et al., 2019; Warnes et al., 2015). PAM analysis performed a principal component analysis (PCA) analyzing

the covariance matrix of the variables and produced a 2D plot which showed the goodness of separation of the gene clusters (which are constructed minimizing the distances between the genes and the designated center points of k clusters). One-way ANOVAs followed by post hoc Tukey's B tests ($p < 0.05$) were used (using the *foreign* and *agricolae* packages in R (R Core Team, (De Mendiburu, 2009)) to assess statistically significant differences between the expression of all genes included in each cluster.

2.4.3. Benchmark doses and point of departures determination

Transcriptomic data obtained in the present study and metabolomic and morphometric data from analogous exposures done in our laboratory (Martínez et al., 2019a; Ortiz-Villanueva et al., 2018) were used to calculate benchmark doses (BMD) per each feature (transcript, metabolite or morphometric trait). In order to do that, measured TBT concentrations were used (see section materials and methods section 2.2). The BMDExpress version 2.2 software (<https://www.sciome.com/bmdexpress/>) was used to carry out this analysis (Kuo et al., 2016; Phillips et al., 2018; Yang et al., 2007). Parameters of each data set (transcriptomic, metabolomic and morphometric) were individually adjusted to a mathematical model (linear, power, Hill, exponential (2nd, 3rd, 4th, 5th order) or polynomial (2nd, 3rd, 4th order)) and the best fitting was identified. The benchmark dose (BMD) of each of these parameters as well as the upper and lower limits (BMDU and BMDL, respectively) were calculated using the best fitting model. Median BMDL for transcriptomic, metabolomic and morphometric datasets was calculated and assigned as the point of departure (PoD; i.e. the concentration from where it can be considered proved the existence of effects of the TBT) as it has been determined as a good estimation for a reference dose (Bhat et al., 2013; Farmahin et al., 2017; Webster et al., 2015).

2.4.4. Gene enrichment analysis

DAVID Bioinformatic Resources 6.8 (<https://david.ncifcrf.gov/home.jsp>) was used for the functional analysis of those genes with an individual BMD value equal or lower to the transcriptomic calculated PoD to assess the first metabolic pathways affected by the TBT. Gene enrichment analysis was performed in DAVID using the default *Danio rerio* background and setting the enrichment significances to a false discovery ratio

(FDR) $\leq 5\%$. Afterwards, DEGs classified in each identified functional module (either from the GO:Biological Function and KEGG (Kyoto Encyclopedia of Genes and Genomes) datasets) were considered separately for the representation of their benchmark dose lower limits. Only pathways with at least five hits were represented, using the *gplots* package in R (R_Development_Core_Team, 2008). KEGG Mapper v4.1 (<https://www.genome.jp/kegg/mapper.html>) was used for the graphical representation of the under/overexpression of the genes involved in the steroid biosynthesis pathway (dre00100).

2.5. Open data

The transcriptomic dataset obtained in this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are freely accessible through GEO Series accession number GSE139599 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139599>).

3. Results

3.1. Survival, hatching and swim bladder inflation

No statistical differences on survival and hatching rates were detected between control and any TBT concentration group at any age. Conversely, a significant decrease in the swim bladder inflation rates were observed in the 56 nM of TBT group at 5 dpf. Complete results are reported in **supplementary table ST1**. The TBT concentration of 56 nM was categorized as the phenotypical LOAEC, as it was the lowest TBT level that exerted any adverse effect over these parameters at any age. This was in agreement with our previous results (Martínez et al., 2019a) and therefore 56 nM was selected as highest tested concentration for the RNA-Seq transcriptomic study.

3.2. Determination and classification of DEGs

ANOVA-PLS analysis identified 3238 transcripts whose mRNA levels changed upon TBT treatment (DEGs) in at least one of the exposure groups (**supplementary table ST2**). Hierarchical clustering of samples closely reflected the experimental setup, as biological replicates for each different treatment group clustered together (**figure F1A**). Remarkably, the highest tested dose (56 nM of TBT) appeared as a clearly

differentiated group from the rest of conditions, including controls. PAM clustering analysis defined the existence of two groups or clusters of DEGs (**figure F1B**). Cluster A (1703 genes) include genes whose relative abundances increased upon TBT exposure, while genes classified in cluster B (1535 genes) showed a decrease in abundance in TBT-treated samples (**figure F1C**). The distribution of normalized expression values (log fold-changes respect to the control group) denotes a clear dose-response pattern, being 56 nM the dose that presented the highest effects when compared to controls (boxplots in **figure F1C**).

3.3. *Analysis of the different points of departure (PoDs) of TBT*

The TBT transcriptomic PoD was estimated to be 9.28 nM (**Figure F2**) using the median BMDL value of the transcripts as described in **section 2.4.3**. It places itself between the low and medium doses used in this study, and it is comparable to the calculated PoD values for metabolite changes (11.48 nM), and well below the corresponding value for morphological traits (67.87 nM) (**Figure F2**). Median of BMD doses (BMD) and BMD upper limits (BMDU) were, respectively, 14.52 and 29.82 nM for transcriptomics, 17.19 and 32.95 nM for metabolomics and 72.97 and 77.01 nM for morphometry. Note the similar slope of the cumulative plot at the three biological levels (**Figure F2**).

Summaries of the best fitted mathematical model for each individual parameter, as well its calculated BMD, BMDL and BMDU values are reported in the **supplementary tables ST3, ST4 and ST5** for the transcriptomic (only DEGs), metabolomic and morphometric data, respectively.

3.4. *Identification of genes involved in molecular initiating events of TBT exposure*

A DAVID functional analysis was performed with those genes with an individual BMD value equal or lower to the transcriptomic PoD (9.28 nM) to assess the first metabolic pathways affected by the TBT. Results obtained after the functional enrichment analysis (**supplementary tables ST6**) showed that most of the affected genes were related to general toxicity and/or pathways related to development: DNA-binding, domain transcription factors, transcription, nucleus, regulation of cell proliferation, cell cycle and differentiation, NAD(P)-binding domain, carbon metabolism, transferases, nucleotide binding, catalytic activity. Averaged BMDL values was estimated for DEGs

included in each of these different functional modules (either from the GO:Biological Function and KEGG datasets), in order to identify molecular initiating events. **Figure F3** shows the distribution of these BMDL values, in which magenta and cyan dots represent the DEGs classified in clusters A (overrepresented) and B (underrepresented), respectively. The results show that functional pathways related to general development were particularly enriched in genes classified in cluster B (downregulated by TBT), while the pathways enriched in DEGs classified in cluster A (upregulated by TBT) were more related with immune and inflammatory response, glutathione metabolism, activation of GTPase activity and tRNA aminoacylation processes (**Figure F3**; note the cell colors). Interestingly, the functional modules with the lowest estimated BMDL were steroid metabolism and biosynthesis pathways, schematically represented in **Figure F4** (map dre00100: steroid biosynthesis pathway, KEGG). The figure shows that genes codifying for the enzymes involved in the synthesis of cholesterol and vitamin D₃, starting at the squalene epoxidation step, were upregulated by TBT at similar concentrations of its PoD (1.7 nM and/or 17 nM of TBT; **Figure F4**; red cells).

4. Discussion

Results from the functional benchmark dose analysis carried out in the present study suggested a general, rather unspecific toxicity pattern of TBT, since most of the affected molecular pathways were related to cell viability and development, except for the specific effect on the steroid metabolism. These results are in agreement with previous studies in fish showing the effect of TBT on DNA-related processes such as DNA repair (Soares et al., 2018; Zuo et al., 2012). On the other hand, our results also showed TBT effects in genes implicated in lipid metabolism and fatty acid biosynthesis pathways, which is consistent with an observed lipidic disruption previously reported in similar experimental setups (den Broeder et al., 2017). However, lipid dysregulation took place only at relatively high TBT concentrations, and it was limited to the transcriptomic level (Berto-Júnior et al., 2018; Grün, 2014; Ouadah-Boussouf and Babin, 2016; Tingaud-Sequeira et al., 2011). The induction of yolk-sac malabsorption syndrome or energy consumption dysregulation by TBT has not been found at this stage of development (5 dpf) (Liang et al., 2017; Martínez et al., 2019a), and

obesogenic effects only appear at later larval stages (15 dpf, (den Broeder et al., 2017)). Interestingly, our results showed that the steroid biosynthesis pathways that lead to the synthesis of cholesterol and vitamin D₃ appeared as the modules that become affected at the lowest TBT concentrations. Since the cholesterol is a key molecule not only for cell membrane formation but also as a precursor for bile acids and steroid hormones synthesis (Anderson et al., 2011; Tokarz et al., 2013), we suggest that TBT exposure could lead important effects over the developing eleutheroembryos. Furthermore, cholesterol and its esters are important components of lipid droplets, which are the preferred form of storage for neutral lipids in the late larval, juvenile and adult stages (Flynn et al., 2009; Minchin and Rawls, 2011). Indeed, those effects in the cholesterol synthesis pathway observed at early stages of the eleutheroembryos development could be the cause of lipid disruptive effects observed later in life, as it has been observed in zebrafish (den Broeder et al., 2017; Lyssimachou et al., 2015). It has been previously reported that vitamin D₃ signaling is crucial for the normal development of the zebrafish during embryo stage, and that its dysregulation can lead a diapause-like arrest (endocrine-mediated developmental delay) in zebrafish embryos and eleutheroembryos (Craig et al., 2008; Romney et al., 2018). This is consistent with our observation that many development-related pathways (cell cycle, mitosis, microtubules, among others) were down-regulated in TBT-exposed embryos. These effects may well be related to previous observation that the morphometric effects of TBT were more related to a developmental delay than to a specific mode of action (Martínez et al., 2019a). Whether or not the developmental delay is mediated by a reduced cell proliferation rate should be further assessed.

A conceivable alternative explanation for the observed effect of TBT may be the release of Sn ion and the subsequent heavy metal poisoning in zebrafish embryos. TBT biodegradation in animal tissues has been suggested to undergo via hydroxylation followed by dealkylation to produce DBT (dibutyltin) and other metabolites, involving mostly cytochrome P450 enzymes (Beyer and Meador, 2011; Nesci et al., 2011). Although these reactions readily occur in zebrafish liver (McGinnis and Crivello, 2011), biotransformation of TBT has not ever been observed in embryos from different fish species, neither TBT biodegradation to inorganic tin ion (Sn^{2+/4+}) (Braunbeck et al.,

1998). Thus, and although we cannot rule out a possible role for a general metal poisoning effects, we propose that TBT exerted its early transcriptomic disrupting effects (mostly in the steroid related pathways) as such, without any significant influence from transformation products. Current evidences indicate the covalent binding of TBT to the retinoid X receptor (RXR) as the molecular initiating event (MIE) that trigger TBT adverse effects in many biological systems (De Voogt, 2015; le Maire et al., 2009). The TBT-RXR complex would form dysfunctional heterodimers with different receptors, including the peroxisome proliferator-activated receptors (PPAR- α , - γ , - δ) or vitamin D receptors (VDR), leading to molecular cascades of reactions that may explain the TBT disrupting properties (Grün, 2014; Huang and Chen, 2017; le Maire et al., 2009; Zhang et al., 2001). These RXRs heterodimers have been suggested to stimulate the steroid biosynthesis in cell cultures (Munetsuna et al., 2009), as well to be related with steroids, organism development, cholesterol, lipid metabolism, fatty acid biosynthesis, and immune response in different animal models (Evans and Mangelsdorf, 2014; Verhaegen, 2012). Therefore, our results are compatible with this proposed MIE for TBT, as all these metabolic pathways appeared as transcriptomically affected by TBT in our study.

Based on the integration of transcriptomic, metabolomic and morphometric data using BMD analysis, we can conclude that TBT exert a rapid and general toxicity in the exposed zebrafish eleutheroembryos. Whereas transcriptomic points of departure of other endocrine disrupting chemicals usually occur at concentrations at least 2-3 orders of magnitude lower than LC_{50} values (Martínez et al., 2019b, 2018; Weber et al., 2013), TBT altered the eleutheroembryo transcriptome and metabolome at only one order of magnitude below the estimated LC_{50} . This is in concordance with the fact that the PoD of TBT for morphological traits (67.87 nM) was found very close to lethal doses (LC_{50} : 93.63 nM; (Martínez et al., 2019a)). Nevertheless, our results support the idea that molecular analysis can be also used as early warning markers for toxicological studies. When comparing BMD results from our study at the transcriptome and metabolome levels, we also observed that both have similar PoDs. It has also been recently demonstrated that short-term transcriptomic PoDs are protective of chronic effects for fish exposed to EDCs, specifically to estrogenic compounds (Pagé-Larivière

et al., 2019). Whereas the use of *-omics* technologies has been applied to study the mode of action and/or the MIE of pollutants (Brockmeier et al., 2017), the application of transcriptomic studies to chemical risk assessment is more limited (Harrill et al., 2019; Thomas et al., 2013). It should be noted in our results the similar slope of the cumulative effect plots of the BMD analysis done with the transcriptomic, metabolomic and morphometric data. As far as we know, not many studies have compared BMD analysis using data from different biological levels. Similar results were obtained in our laboratory in zebrafish eleutheroembryos exposed to PFOS when comparing transcriptomic and morphometric parameters using the BMD approach (Martínez et al., 2019b). We consider at least possible that the similar slopes of both curves may reflect the propagation of molecular events (transcriptome and metabolome) to higher levels of organization (morphology), although this assumption should be further explored. In summary, our results highlight the value of transcriptomic data and of the use of transcriptomic dose-response studies in environmental risk assessment.

A significant advance in the use of genomic data into risk assessment is due to the development of methods that integrate BMD approaches to analyze transcriptomic data. This method allows the identification of reference doses at which particular cellular responses are affected (Thomas et al., 2007). The study of the transcriptomic effects of TBT at the functional level using the BMD approach, instead of looking directly at the functionality of the DEGs, can also prevent the lack of reproducibility of specific identified DEGs as biomarkers between experiments or the lack of linkages with the phenotype, as have been observed in some clinical studies (Myers et al., 2015; Song et al., 2018). This is so because the effects of a pollutant on functional pathways are generally more robust than their effects on the expression of specific genes, whatever useful may they be for exposure and effect assessment studies. In addition, the application of benchmark dose approach in toxicology, chemical effects and risk assessment are nowadays preferred to other more conventional parameters as NOECs or LOECs (Altenburger et al., 2012; Escher et al., 2014; Mayfield and Skall, 2018) and allow us to compare different datasets at different doses, as previously reported (Tu et al., 2019). Finally, applying the BMD concept at the functional level

allowed us to separate the initially affected pathways (steroid biosynthesis, in our case) from the ones disrupted at higher concentrations (cell viability, general development or lipid-related pathways, among others).

5. Conclusions

In summary, the present dose-response transcriptomic analysis allowed us to determine the most sensitive pathways affected by TBT, mostly related to the steroid biosynthesis, general cell viability and development. The transcriptomic effects in genes related to general toxicity may be related to a developmental delay induced by relatively high TBT concentrations, in agreement with previous morphometric results. Obesogenic effects, traditionally attributed to TBT, only appeared at concentrations relatively close to lethal levels. This is different from the typical pattern of toxic effects for most EDCs, like estrogenic compounds or thyroid disrupters, in which the earlier endocrine effects appear at concentrations two or three orders of magnitude lower than the LC_{50} suggesting that the toxic mode of action of TBT could differ from other EDCs. The present work thus contributes in the understanding of the environmental hazards associated to TBT and highlights the utility of using the benchmark dose approach with *-omic* datasets at the functional pathway level in environmental risk assessment.

Authorship contribution statement

- Rubén Martínez: Conceptualization; Data curation; Formal analysis; Investigation; Visualization; Writing - original draft; Writing - review & editing;
- Anna E. Codina: Data curation; Methodology;
- Carlos Barata: Funding acquisition; Writing - review & editing;
- Romà Tauler: Funding acquisition; Writing - review & editing;

· Benjamin Piña: Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Supervision; Visualization; Writing - original draft; Writing - review & editing;

· Laia Navarro-Martín: Conceptualization; Data curation; Formal analysis; Investigation; Project administration; Supervision; Visualization; Writing - original draft; Writing - review & editing;

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Figure Legends

Figure F1

Figure 1. A) Heatmap corresponding to the 3238 transcripts identified as differentially expressed genes (DEGs) by ANOVA-PLS analysis. Represented values were normalized, centered to average values of control samples and then log2 transformed. Color scale ranges from blue (underrepresented DEGs) to red (overrepresented DEGs); white cells correspond to values identical to the average values of the controls. Both genes (rows) and samples (columns) were grouped by hierarchical clustering, illustrated by the dendrograms at the left and the top of the panel, respectively. B) PCA analysis from PAM clustering of DEGs, showing the two defined clusters (cluster A, in magenta, and cluster B, in cyan). The two first components explained the 61.73% of total point variability. C) Individual heatmaps of the DEGs classified by the PAM clustering in each cluster and distribution of their normalized abundance values. Low-case letters at the top of each group indicate statistically different distributions (determined by an ANOVA followed by a Tukey's B ($p \leq 0.05$) post-hoc test. Colored boxes included values

between the 1st and 3rd quartiles, means are indicated as thick bars and whiskers cover the total distribution, except for outliers (represented as circles).

Figure 2. Accumulation plot of BMD values for the parameters of the different biological levels of zebrafish eleutheroembryos exposed to TBT: transcriptomics (in blue), metabolomics (in purple) and morphometrics (in orange). Points represent the number of parameters with a BMD value lower than a specific TBT concentration. The best BMD value for each DEG/metabolite/morphological parameter was determined using the BMDExpress version 2.2 software (<https://www.sciome.com/bmdexpress/>) (Kuo et al., 2016; Phillips et al., 2018; Yang et al., 2007). The median BMDL values were used as PoD (point of departure) for the three biological levels and are interpolated in the graph as dotted lines. Green dotted line indicates the 100% of the studied parameters for each level (3238 differentially expressed genes, 55 metabolites and 9 morphological parameters).

Figure 3. BMDL values for individual DEGs, classified by their annotation to functional modules. For each module, grey box includes values between the 1st and 3rd quartiles and a thick bar indicates the mean. Calculated BMDL of DEGs classified in cluster A and B are represented as magenta and cyan points, respectively. For helping visualization, green vertical line indicates the median BMDL considered as the transcriptomic point of departure (PoD) for TBT exposure (**figure 2**). Green dotted horizontal line separates the functional modules with median BMDL values higher or lower than global transcriptomic PoD. On the right, distribution of DEGs among the different functional modules (rows) and clusters (columns). Numbers express the absolute number of DEGs in each functional module and cluster, box colors indicate the relative importance of DEGs associated to each pathway for each cluster (from light-yellow -most- to red -less-; two cells of the same color correspond to an identical relative fraction of DEGs in both clusters). For simplification, only functional modules with at least 5 DEGs in at least one of the clusters were represented. Redundant functional modules were simplified to the one with the highest number of hits.

Figure F4

Figure 4. Graphical representation of the steroid biosynthesis pathway (dre00100, Kyoto Encyclopedia of Genes and Genomes). Grey cells represent genes not present in zebrafish. Genes colored in white did not show changes on their mRNA levels in the 1.7 or 17 nM TBT exposure groups, those that showed overexpression in at least one of these concentrations are colored in red (8 genes in total). Those concentrations were chosen to represent the behaviour of the genes at concentrations close to the transcriptomic PoD (9.28 nM of TBT).